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# Assesment of pMTL Construct for Detection *in vivo* of Luciferase Expression and Fate of the Transgene in the Zebrafish, *Brachydanio rerio*

Jawahar G. Patil, Veronica Wong and Hong Woo Khoo

*Fisheries Biology Laboratory, Department of Zoology, National University of Singapore, 10, Kent Ridge Crescent, Singapore-0511*

**ABSTRACT**—Early embryos of the zebrafish *Brachydanio rerio* were cytoplasmically microinjected with pMTL plasmid containing firefly luciferase gene, in both linearized- and supercoiled-plasmid forms, to evaluate *in vivo* expression, pattern of integration and germ-line transmission of the transgene in the host fish. It was possible to detect luciferase expression *in vivo*, and the pattern of time-course expression was similar in both linearized- and supercoiled-plasmid injected groups. Strong luciferase activity was detected 15–20 hours after injection, coinciding with early somitogenesis. Expression was detectable in a few 1 week-old individuals but was not detectable in all adults and in $F_1$ progeny. *In vivo* screening for expression of the transgene in the developing embryo using luciferase assay as a method for detecting the presence of the transgenic fish compares favourably, with PCR and Southern blot analysis (SBA). No integration of the introduced DNA into the genome of treated fish and their progeny, was detected, instead it remained in extrachromosomal form. Most of the first generation founders were mosaic. Germline transmission was observed in one individual only. A probable reason for the absence of integration in this study when compared to the varying frequencies of integration reported earlier in the same fish is discussed.

## INTRODUCTION

Considerable research and progress has been made in the production of transgenic fish in recent years [for review see 7]. To demonstrate the occurrence of transgenic animals, tedious methods such as PCR and Southern blot analysis (SBA) have been used, followed by genetic crossing to isolate the required phenotype. The PCR and SBA techniques often require sacrifice of the animals to extract the DNA. Such invasive procedures preclude subsequent breeding experiments on germ-line transmission. Alternatively, treated individuals are grown to large size and part of the animal such as fin sample were used for assay. Raising fish to the adult stage is, however, time consuming and expensive. A more expeditious method for detecting transgenes is therefore required.

This study investigates the use of firefly luciferase as a reporter gene for convenient detection of the presence of the transgene in zebrafish *in vivo*. The sensitivity, rapidity, non-invasiveness and use of this non-radioactive technique for positive indication of presence, when compared to other commonly used reporter genes such as CAT, make this luciferase assay a more efficient one. A few reports on the use of firefly luciferase gene as reporter in fish are available [1, 6, 19]. Sato *et al.* [14] have shown the expression of luciferase in medaka, using tissue lysate. This study investigates the efficiency of luciferase expression *in vivo* as well as the fate and outcome of the introduced pMTL reporter with regard to its integration into the fish genome and its germ-line transmission.

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## MATERIALS AND METHODS

We have used the zebrafish in this study. The zebrafish stock was bought from local suppliers and fish were raised in aquarium tanks. The pMTL plasmid used in this study was earlier used by Sato *et al.* [14] in medaka. It contains Chinese hamster metallothionein I (MT-I) promoter, the cDNA for luciferase from the firefly (*Photinus pyralis*), the SV40 polyA tail and the vector sequence of pBR322. The construct was a gift from Dr. T. Ishikawa, Dept. of Experimental Pathology, Tokyo University.

The method of microinjection described by Khoo *et al.* [8] was followed, with slight modifications. About 1–2 nl of a solution of Hind III-linearized or supercoiled-plasmid DNA (100 $\mu$g/ml DNA in 0.1 M Tris-HCl, pH 7.2, containing 0.25%, w/v, phenol red) were injected into embryos at the one or two-cell stage (about $1.4\times10^7$ copies/embryo), with a sharpened glass needle (3–5 $\mu$m in diameter). Injection volume was controlled with an automatic microinjector (Model IM-1, Narishige, Tokyo).

To monitor the expression of luciferase, embryos were immersed in 100 $\mu$l of luciferase assay reagent (LAR; Promega, Madison, USA.) and luminescence was detected with a liquid scintillation counter (model LS 5801; Beckman.) in the single-photon monitor mode. For subsequent screening of expression in individual live embryos, the counting was performed in 60 $\mu$l of LAR+40 $\mu$l of distilled water, at about 15 hr after microinjection. For adult fish fin samples were washed twice in PBS ($Ca^{2+}$ and $Mg^{2+}$ free); a quarter of the fin was assayed intact by immersion of the tissue in 100 $\mu$l LAR and another quarter was homogenised in 100 $\mu$l of 1X lysis buffer (supplied by the manufacturer; Promega.) and then assayed with 100 $\mu$l of LAR. Remaining half of the fin sample was used for DNA extraction.

To extract genomic DNA, each fry or fin was rinsed in embryonic solution containing 10 $\mu$g/ml of DNAse I for a period of 1 hr and washed 4× in PBS and once in Tris-EDTA (10 mM Tris; 1 mM EDTA). The fry was digested in 25 $\mu$l of polymerase chain

reaction (PCR) buffer supplemented with nonionic detergent and proteinase K (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.025 mg gelatin, 0.45% NP 40, 0.45% Triton X-100 and 60 $\mu$g/ml proteinase K) at 55°C for 90 min and heat inactivated at 95°C for 10 min. The digested mixture was then cleared by centrifugation (3000 g for 3 min). An aliquot of 2 $\mu$l was used for PCR. The remaining 23 $\mu$l was purified by phenol: chloroform: isoamyl alcohol extraction. DNA from adult fish was extracted as described elsewhere [9].

Two oligonucleotide primers 5′-CGGCGGCGGGAAGTTCA-CCGGCG-3′ and 5′-CCGGGCGCGGTCGGTAAAG-3′ were used for detection of the firefly gene for luciferase. PCR was carried out following the method of Saiki [13] with slight modification. The crude genomic extract (2 $\mu$l) was placed in 0.5 ml microfuge tube containing PCR mixture (1X PCR buffer containing 50 $\mu$M each of dATP, dCTP, dGTP, dTTP, 0.25 $\mu$M each of primer and 0.25 unit of Taq DNA polymerase). The entire reaction mixture was overlaid with mineral oil and amplified in a Techne PHC-2 thermal cycler (Princeton, USA) for 30 cycles. Amplification was performed with initial denaturation at 94°C for 5 min, with subsequent incubations at 95°C, 55°C and 72°C for 1, 1.3, and 1 min, respectively. Then 8 $\mu$l of the amplified mixture were mixed with 2 $\mu$l of loading buffer and subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light.

The remaining 23 $\mu$l of the genomic DNA extract were digested by Hind III (New England Biolabs, Beverly), subjected to electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N (Amersham, UK.) nylon membrane by vacuum blotting using a trans-Vac TE 80 system (Hoefer Scientific Instruments, San Francisco). Membranes were probed with Hind III linearized plasmid that had been labelled with [$^{32}$P]-dCTP (Amersham, UK.) by random priming. Hybridization was performed at 65°C overnight. Filters were then washed and autoradiographed using Kodak (X-OMAT) diagnostic film. In case of adult fishes where there was sufficient genomic DNA extract, 5 $\mu$g was HindIII digested and another 5 $\mu$g undigested for SBA.

Three experiments were conducted. The first was an *in vivo*-time course expression assay of zebrafish embryo treated with pMTL plasmid. Microinjected embryos in duplicates of 5 were monitored for expression in each treatment groups. Embryos injected with injection buffer only and those not injected served as injected and non-injected controls, respectively.

In the second experiment we tested whether the presence of the transgne could be detected by PCR and Southern blot in all the embryos expressing the transgene *in vivo*. About 400 embryos were microinjected with supercoiled- and linear-plasmid separately. Embryos which gave more than 1.5 times the background counts at 10–15 hr after injection were considered as positive for expression. Three days after hatching, 200 each hatchlings from the treated groups were sampled for PCR and Southern blot analysis.

The third experiment was to conduct a germ-line transmission study. Embryos microinjected with supercoiled- and linear-plasmid, were screened for luciferase expression and expression positive individuals were reared to maturity. All the individuals whose fin samples were positive for both PCR and SBA (henceforth referred to as fin-positives) were then bred with untreated fish to produce $F_1$. The $F_1$ progeny were first tested by luciferase expression assay and subsequently the same samples were tested by PCR and SBA. Similarly, some individuals whose fin samples tested negative by PCR and SBA (fin-negatives) were also bred with untreated fish and their progeny assayed as above.

## RESULTS

*Experiment I*

The time-course expression of luciferase in the developing embryos is presented in Figure 1. Scintillation counts recorded from the sample blank and the control groups were not significantly different ($P<0.05$) from one another. Both injected groups (supercoiled and linear) gave significantly higher scintillation counts ($P<0.05$) than the controls and the blanks. The results indicate that the luciferase expression in injected embryos increased rapidly to a maximum at 15 hr after injection coinciding with early somitogenesis. The first evidence of expression was obtained 10 hr post-injection and this coincided with the late gastrula stage. Subsequently, the counts declined gradually but still was detectable 48 hr post-injection. The decline in expression was more rapid in supercoiled-plasmid injected group than in the linear-plasmid injected group. Some fry (3 days old) gave scintillation counts similar to those observed at 48 hr, but no values higher than those at 15–25 hr after injection. Expression in such fry persisted for up to 8 days. Since, in preliminary experiments, we neither detected endogenous luciferase activity nor any evidence of inhibitory factors, the observed pattern of luciferase activity must represent luciferase expression by the introduced gene.

*Experiment II*

Forty nine of the 200 (24.5%) inidividuals in the supercoiled-plasmid injected group were found to be positive for expression (Table 1). Similarly, 37 individuals out of 200 (18.5%) in the linear-plasmid injected group were positive for expression (Table 2). All individuals that expressed the transgene were also positive by PCR and Southern blot, in both treatment groups. The remaining 75.5% and 81.5% in the supercoiled- and linear-plasmid treated groups, respectively, did not express the inserted gene. These non-expressing embryos were also PCR and Southern blot-negative except for a few individuals i.e. 4% in supercoiled-plasmid and 3% in linear-plasmid injected groups were PCR-positive, whereas 2% in the supercoiled-plasmid and none in linear-plasmid treated groups were Southern blot-positive. Typical examples of PCR results for the supercoiled group are shown in Figure 2.

All the 26.5% and 18.5% SBA positive individuals in the supercoiled- and linearized-plasmid injected groups respectively were invariably PCR-positive. However, the inverse was not true as 2% and 3% of the individuals in supercoiled and linear-plasmid injected groups respectively were PCR-positive but Southern blot-negative. Southern blot profile (Fig. 3) of 11 fry from the supercoiled-plasmid injected group that were PCR-positive showed only one band (lanes 2–11) at the size of linearized-plasmid (6.65 kb) suggesting that, there was no genomic integration of the introduced gene. SBA of fry from linear-plasmid injected group gave similar results (Data not shown).

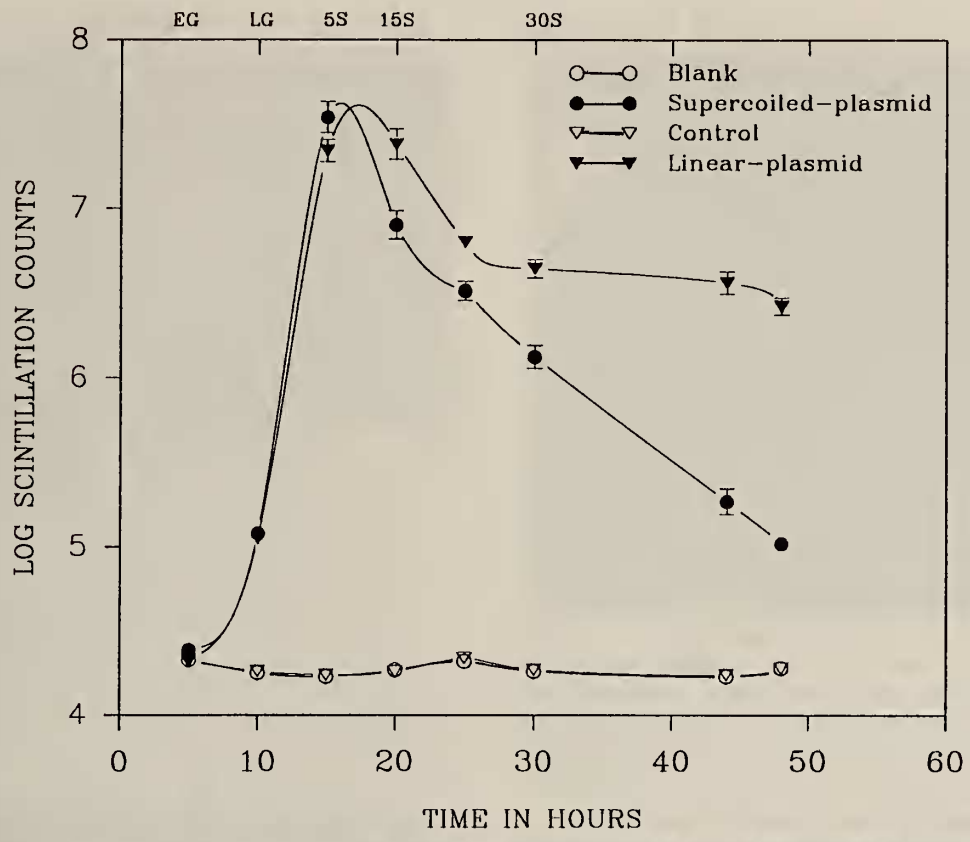


FIG. 1. Time course of expression of the luciferase gene after microinjection into zebrafish embryos as measured with a scintillation counter (in the single-photon counting mode). Hours after microinjection are indicated on the abscissa. Above the figure several events in the development of the fish are indicated. (EG, early gastrula; LG, late gastrula; 5s, 15s and 30s, correspond to 5-, 15- and 30-somite stages respectively.) Scintillation counts are expressed logarithmically ($\log_{10}$).

TABLE 1. Frequency of the transgene in the supercoiled-plasmid microinjected group

| | | PCR | |
|---|---|---|---|
| | | % Positive | % Negative |
| Expression | % Positive | 24.5* (49) | 0 |
| | % Negative | 4 (8) | 71.5 (143) |
| Southern blot | % Positive | 26.5 (53) | 0 |
| | % Negative | 2 (4) | 71.5 (143) |

* Southern blot positive as well.
Figures in parenthesis indicate number of individuals out of the 200 assayed.

TABLE 2. Frequency of the transgene in the linear-plasmid microinjected group

| | | PCR | |
|---|---|---|---|
| | | % Positive | % Negative |
| Expression | % Positive | 18.5* (37) | 0 |
| | % Negative | 3 (6) | 78.5 (157) |
| Southern blot | % Positive | 18.5 (37) | 0 |
| | % Negative | 3 (6) | 78.5 (157) |

* Southern blot positive as well.
Figures in parenthesis indicate number of individuals out of the 200 assayed.

*Experiment III*

Of the 36 individuals in the supercoiled- and 29 in the linear-plasmid injected groups which grew to maturity, eight and five individuals, respectively, were both PCR and SBA positive (Table 3). None showed expression of the luciferase gene. The SBA profile obtained from fin samples of 8 SBA positive individuals of supercoiled-plasmid treated group are shown in Figure 4. In all eight individuals only one band about the size of the linearized plasmid (6.65 kb) was detected (lanes 2–9). This suggests that the injected

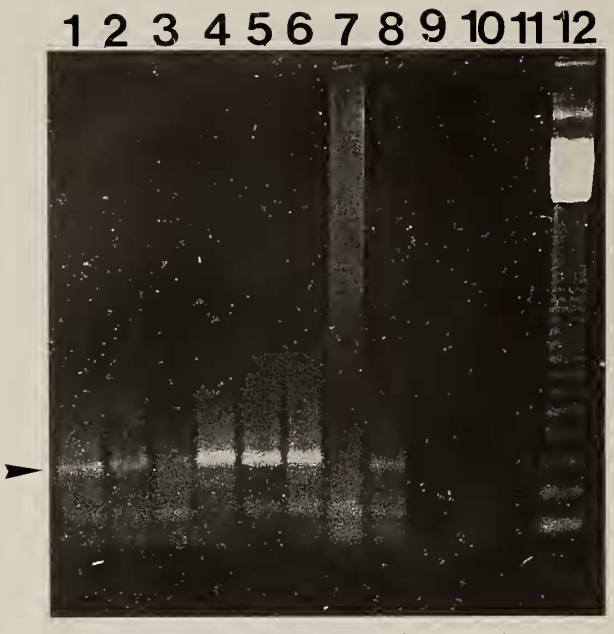


Fig. 2. Detection by PCR analysis of foreign DNA in transgenic zebrafish that had been injected with supercoiled plasmid. 8 $\mu$l of DNA from the total reaction mixture after PCR were fractionated on a 1.5% agarose gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. Lanes 1–3 and 7–9, amplified genomic DNA from expression-negative individuals; lanes 4–6, amplified genomic DNA from expression-positive individuals; lane 10, amplified genomic DNA from an untreated control fish; lane 11, reagent blank and lane 12, ladder. Arrowhead indicate the expected, amplified 376-bp bands.


Fig. 3. Southern blot analysis of supercoiled-plasmid injected group. Genomic DNA of individuals that had been assayed for expression and by PCR were digested with Hind III (single restriction site) then the entire aliquot was subjected to electrophoresis on a 0.8% agarose gel, transferred to a Hybond-N membrane and probed with [$^{32}$P]-rediolabelled pMTL (Hind III-linearized). Lane 1, $\lambda$ DNA/HindIII size markers; lanes 2–12, genomic DNA of the individuals that were PCR positive; lane 13 genomic DNA of control fish; lane 14 genomic DNA of control fish spiked with pMTL (positive control). Arrowhead indicate to the size of the linearized plasmid.

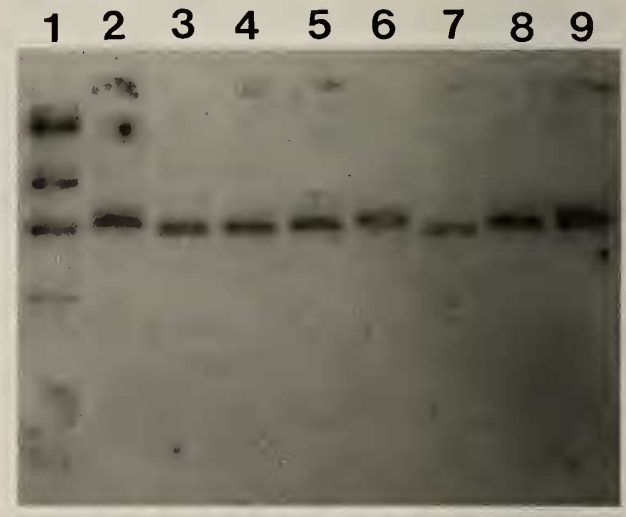


Fig. 4. Detection of transgene in $F_0$ of supercoiled-plasmid injected group. Genomic DNA (about 5 $\mu$g) extracted from fin clip of adult fish (that were PCR positive) were Hind III digested and analyzed by Southern blot as in legend to Fig. 3. Lane 1 $\lambda$ Hind III marker; lane 2–9 genomic DNA from 8 different adult $F_0$ fish that were earlier found PCR positive.

reporter gene remained unintegrated with the fish genome. None of these fin-positive individuals transmitted the transgene to their offspring ($F_1$), as their offspring were neither expression-, PCR- nor Southern blot-positive (Data not shown).

One of the fin-negative individuals in the supercoiled-plasmid injected group, transmitted the foreign DNA to its offspring. The transmission was evident from SBA profiles of 4 $F_1$ fish (1 month old), resulting from a mating between the SBA negative founder and a control fish (Fig. 5). All the four $F_1$ fish tested had similar Southern blot signal patterns that hybridized with the plasmid probe. The inherited gene was about 2.48 kb in size following digestion with Hind III (lanes 1, 3, 5 and 7). The undigested genomic

Table 3. Transgenic adult fish after injection of supercioled- and linear-pMTL DNA

| | | Supercoiled plasmid injected group | | Linear plasmid injected group | |
|---|---|---|---|---|---|
| | | Southern blot | | Southern blot | |
| | | No. Positive | No. Negativc | No. Positive | No. Negative |
| PCR | No. Positive | 8 | 0 | 5 | 0 |
| | No. Ncgativc | 0 | 28 | 0 | 24 |

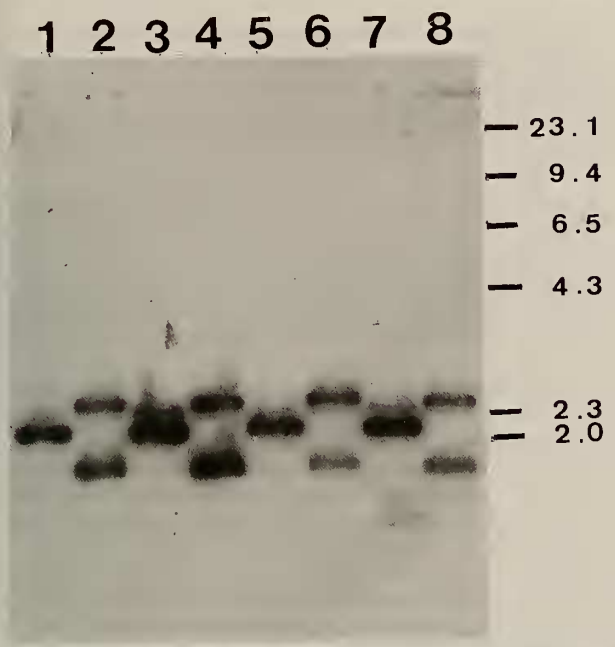


FIG. 5. Southern blot analysis of $F_1$. Genomic DNA (about 7 $\mu$g) from whole fish of four individuals from the progeny of a founder crossed to control fish was either Hind III digested or undigested and probed (please see legend to Fig. 3.). Fin clip assay of the founder was negative for both PCR and Southern blot. Lanes 1, 3, 5 and 7, Hind III digested samples; lanes 2, 4, 6 and 8, undigested samples.

DNA showed two bands of about 2.4 kb and 1.9 kb (lanes 2, 4, 6 and 8). This pattern suggests that a portion of the introduced plasmid had been excised out. PCR gave negative results for the four samples ($F_1$ fish), supporting the hypothesis that the deletion included a portion of the luciferase fragment in the introduced plasmid. Higher intensities of the Hind III linearized band in all four analyzed samples when compared to band 1 in undigested samples suggest that the transgene remained extrachromosomal in two different forms.

## DISCUSSION

We have demonstrated that it is possible to detect the *in vivo* expression of firefly luciferase in zebrafish embryos nondestructively. The LAR penetrates easily into the embryos and larvae and does not seem to harm the developing embryos. This not only facilitates monitoring of the reporter gene activity during development but also the detection of foreign DNA from the same embryo. Papp *et al.* also detected *in vivo* expression of the luciferase gene in zebrafish embryos injected with another plasmid, pCMV1 containing the luciferase gene (unpublished data). The expression of the luciferase gene was transient with the onset of expression coinciding with the late gastrula stage. This onset differs from that observed by Chong and Vielkind [4], who found in the medaka, that the onset of CAT (pUSVCAT) expression occurred prior to gastrulation, at the flat-blastula stage. However, they reported that onset of expression was 10 hr post-injection, similar to the time of first detection in the present study. The difference in stage dependent expression could be due to species differences. For both the supercoiled- and linear-plasmid DNA forms, the luciferase time-course expression patterns were very similar, though not identical. These results indicate that the physical conformation of the administered DNA does not appear to affect its expression.

PCR seemed slightly more sensitive than detection of luciferase expression as a method for detection of the transgene. Nevertheless, the ability to detect the expression in embryos without destroying them (non-destructive) and the rapidity of the assay favour the latter method, aiding both in the rapidity of screening and in limiting the rearing of founders to maturity.

Our Southern analysis of the genomic DNA of several transgenic fish microinjected with supercoiled-plasmid indicated no integration of the foreign DNA. Rather the introduced DNA remained extrachromosomal. Extrachromosomal occurrence and inheritance of transgenes in *Caenorhabditis elegans* [11, 15], mice [12, 18] and zebrafish [2, 9] have been previously reported. Integration of the introduced foreign genes in zebrafish have been earlier reported [2, 5, 16, 17].

The inability of the fin positive parents to transmit the transgene to their offspring suggests that germ cells of these tested individuals did not contain the transgene. Whilst the ability of one of the fin-negative individual to transmit suggests that the majority of the founders are mosaics. Mosaicism is consistent with the earlier observations made in this fish [2, 5, 16, 17].

Occurrence of the inherited transgene in smaller fragments than the plasmid in the $F_1$ of the fin-negative founder suggests that a part of the transgene was excised. Furthermore, inability of PCR to detect the inherited gene implies that the deletion encompassed the luciferase gene. Deletion of integrated transgenes in mice have been observed by Komori *et al.* [10] and Bluthmann *et al.* [3].

The discrepancy between the low frequency of germ-line transmission and absence of integration observed in the present study, and that of 4–5% [16, 17] and 17% [5] reported previously in this species require further clarification; however these differences may be attributable to the nature of the plasmid constructs. In the studies of Stuart *et al.* [16, 17] and Culp *et al.* [5], the plasmid constructs (pUSVCAT and pRSV-$\beta$Gal, respectively) contained RSV-LTR enhancer/promoter sequences. In contrast the pMTL construct used in this study had MT-I promoter and SV40 poly A tail, which for unknown reasons seems to have lower tendency to integrate or no integration at all in this fish. This argument is further supported by the fact that following injection of the plasmid construct SV40-*lacz* (pCH110; that lacked RSV-LTR fragments), containing the same reporter as in pRSV-$\beta$Gal, Culp *et al.* [5] also did not detect any germ-line transgenic fish.

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